ZOOLOGICAL SCIENCE 6: 715–719 (1989)

The Alkaline Substances and Other Constituents of Blastocoel Fluid of the Newt Embryo

Tezro Asao

St. Marianna University, School of Medicine, Biological Laboratory, Miyamae-ku, Kawasaki 213, Japan

ABSTRACT—Blastocoel fluid of early gastrula of the newt, *Triturus pyrrhogaster*, was initially separated into four fractions by gel permeation chromatography. The estimation or chracterization of the fractions was performed by infrared spectrometry or SDS-polyacrylamide gel electrophoresis. The alkalinity of blastocoel fluid (pH 8.83 to 9.19) was owing to the existance of ca. 40 mM of sodium bicarbonate and sodium carbonate. Blastocoel fluid also contained small, acidic molecular carbonates including amino acids, which seemed to buffer blastocoel fluid against salts of sodium carbonate. Over twenty kinds of protein were countable by silver stain method after SDS-PAGE. Concentration of inorganic ions was 72 mM in Na^+, 6.9 mM in K^+, and 1.3 mM in Ca^{++}.

INTRODUCTION

At the gastrula stage a dramatic change begins, namely, the presumptive mesodermal and endodermal tissues invaginate the blastocoelic cavity and form a double-ball type embryo. This is the beginning of morphogenesis. However, although many studies have been performed on the inductive experiments [1, 2], there is as yet little information concerning the blastocoel fluid of the newt embryo. Although the intercellular pH of the morulla embbryo of *Xenopus* has been reported to be 8.4 [3], the blastocoel fluid of newt gastrula also has shown alkalinity in our preliminary experiments. The constituents of the blastocoel fluid of newt gastrula are analyzed and discussed in this paper.

MATERIALS AND METHODS

Newt eggs were obtained by injection of 50 μl gonadotropin solution per day (75 units, Teikokuzoki Seiyaku, Tokyo) into female newts for five days. The female newts were commercially gathered at several places in the Tohoku districts. Eggs reached early gastrula on the third day at 18°C. Capsules of embryos were removed manually by gentle use of Wickel's scissors. The embryos were then set in small holes made on an agar bed in a glass dish. A micro-capillary tube connected to a micro-syringe handled by micro-manupulater was advanced into the blastocoel of early gastrula through a vitelline membrane and an outer blastoderm. Usually, at most 2 μl of blastocoel fluid was sucked out into the capillary tube per embryo by reversal usage of the syringe. The fluid was collected in a plastic micro-centrifuge tube with a cap. The tube was centrifuged at 1,800×g for five minutes. Supernatant was used as blastocoel fluid in the experiments leading to this paper. About 40 μl of mixed blastocoel fluid from different embryos was used to obtain a measurement by using a single complex micro-electrode (Microelectrode Company, New Hampshire, USA).

One hundred μl of mixed fluid was used in a measurement of inorganic ions analysis. Sodium and potassium ions were analyzed by atomic absorption spectrometry, calcium ions by ICP emission spectrometry, chlorine ions by ion chromatography.

The constituents of blastocoel fluid was analyzed by gel permeation chromatography, using Cellulofine GCL 25 superfine (Seikagaku Kogyo, Tokyo). The blastocoel fluid (0.3 ml) was applied to the column (1.5 cm Ø, 48 cm in length) and was

Accepted September 19, 1988
Received July 2, 1988

eluted out with distilled water at a velocity of 1 ml per 6 min. Each fraction (1 ml) was monitored at 200 nm and its pH was measured. The peak fractions in question were collected and lyophilized for infrared spectrometry or electrophoresis.

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [4], though with some modifications. Gradient slab gel (14×14×0.1 cm) was prepared from a stock solution of 30% acrylamide and 0.8% methylene bisacrylamide. The final concentration of separation gel were 0.375 M Tris-HCl containing 0.1% SDS (pH 8.8), and 7.5 to 18% acrylamide. The gel was polymerized by the addition of 0.025% tetramethylethylenediamine and 1% ammonium persulfate. The stacking gel concentration was 0.375 M Tris-HCl (pH 6.8), 0.1% SDS and 3.5% acrylamide. Each preparation in water (10 to 50 μl) was mixed with an equal volume of the sample solution containing 0.125 M Tris-HCl (pH 6.8), 4% SDS, 0.002% bromophenol blue in ethanol, 10% β-mercaptoethanol and 20% glycerol. Proteins were completely dissociated by immersing the tubes in boiling water for three minutes. Electrophoresis was continued under constant current of 25 mA till the marker dye reached to 2 cm from the margin. The proteins in gel were detected by the silver stain method using a Silver Stain Kit (Wako Pure Chemical, Tokyo).

Infrared spectrometry of the lyophilized specimen at its third and fourth peaks were measured by the FT-IR diffuse reflectance method.

RESULTS

Blastocoel fluid was fairly alkaline. As shown in Table 1, pH values varied from 8.83 to 9.19. The variety of the pH values was not due to error in the measurement, but to differences of habitat in the female newt group.

Table 1. pH values of blastocoel fluid

Group	pH (mean value±S.D.)
1	8.83±0.05 (n=10)
2	9.01±0.06 (n= 5)
3	9.19±0.06 (n= 4)

Mixed blastocoel fluid (about 40 μl) collected from about 30 embryos was used per a measurement of pH.

The inorganic ion contents in blastocoel fluid are shown in Table 2. Concentrations of sodium, potassium, and calcium ions were 72, 6.9 and 1.3 mM, respectively. Values of the concentrations of

Table 2. Inorganic cation and chlorine ion concentration of blastocoel fluid

Ion	Mean concentration (mM±S.D.)
Na^+	71.7±2.2 (n=3)
K^+	6.9±1.5 (n=2)
Ca^{++}	1.2±0.3 (n=2)
Cl^-	42.3 (n=1)

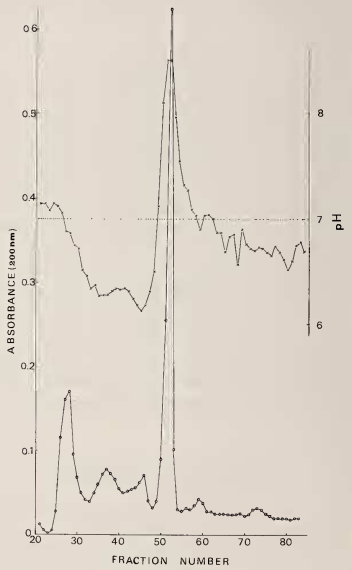

Fig. 1. Gel permeation chromatography of blastocoel fluid.

The gross blastocoel fluid (0.3 ml) was applied. The conditions of chromatography are mentioned in the text. Absorbance is indicated by a small circle and pH, by a cross. The fourth peak was alkaline while the second and third peaks were acidic.

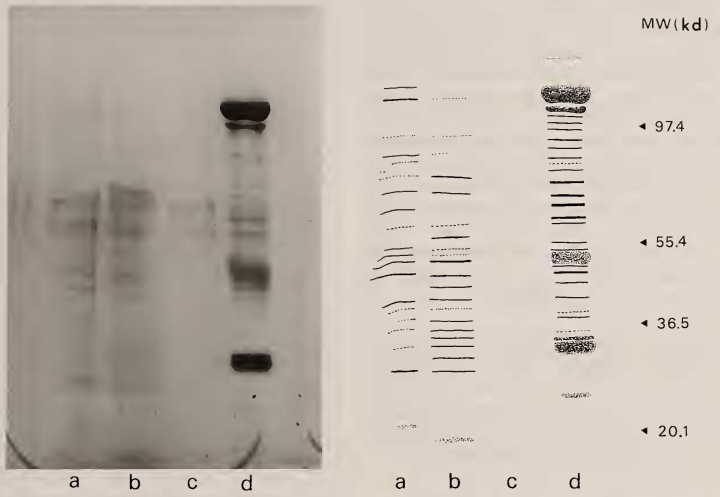

FIG. 2. Polyacrylamide gel electrophoresis in SDS (left), and sketches (right) of the gross blastocoel fluid, its subcomponents separated by chromatography and homogenate of embryo.

The lanes a, b, c and d are, gross blastocoel fluid, first peak, second peak in chromatography and homogenate of embryo, respectively. Two bands appeared commonly in the lanes a, b and c are contaminations from the reagents used.

potassium and calcium ions, however, had large standard deviations.

Gel permeation chromatography of the blastocoel fluid gave rise to four peaks (Fig. 1). As mentioned in the Methods and Materials section above, the first peak at void volume was further analyzed by SDS-polyacrylamide gel electrophoresis. The pH measurement of each fraction showed that the second and third peaks wer acidic, while the fourth peak was alkaline. The third and

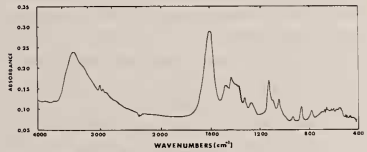

FIG. 3. Infrared spectrum of the third peak in chromatography.

Absorptions at 1400 and 1600 cm^{-1} revealed carbonic salts. A broad band of absorption from 2500 to 3600 cm^{-1} suggested the existance of NH or OH structure.

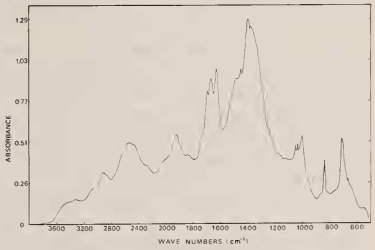

FIG. 4. Infrared spectrum of the fourth peak in chromatography.

The spectrum of the fourth peak was very similar to that of sodium bicarbanate but for the absorption at 1400 cm^{-1}.

fourth peaks were further analyzed by infrared spectrometry.

The SDS-polyacrylamide gel electrophoresis pattern of gross blastocoel fluid, together with the first and second peaks in chromatography and the homogenate of embryos are shown in Figure 2. From the electrophoresis band patterns of the first peak and the gross blastocoel fluid, at least twenty kinds of protein were possibly detected, of 17.6, 27, 29, 31, 33.5, 35.4, 37, 39, 40, 42, 44, 47.5, 49, 51, 53, 65.5, 72.5, 80.5, 89, 105 and 114×10^3 daltons, although some bands existed only in the gross blastocoel fluid. On the other hand, any bands were scarcely recognizable in the lane of the second peak.

Infrared spectrums of the third and fourth peaks are shown in Figures 3 and 4, respectively. Absorption at 780, 860, 930, 1045, 1122, 1267, 1317, 1427 and 1473 cm^{-1} were recorded in the third peak and in the fourth 700, 850, 1000, 1400, 1630, 1700, 1930, 2500-2600, and 2900 cm^{-1}.

DISCUSSION

The alkalinity of the blastocoel fluid of newt gastrula is possibly due to the existance of both sodium bicarbonate and sodium carbonate. The reasoning is as follows: firstly, the infrared spectrum of the fourth peak in gel permeation chromatography was similar to that of sodium bicarbonate, whose absorption peaks exist at 680, 820, 990, 1290, 1620, 1920, 2500 etc. cm^{-1}. On the other hand, the absorption peaks of sodium carbonate are at 700, 880, 1410–1450, 1780 etc. cm^{-1}. The spectrum of the mixture of these two salts was therefore closer to that of the fourth peak (see results). Secondly, the chromatography of the mixture of both salts under the same conditions could successfully simulate that of fourth peak in the elution pattern and the pH value of each of its fractions (Fig. 5). The sharpness of the peak is owing to the coexistance of other components, while the shift in the elution position seemed to be owing to the concentration of the applied salt mixture. In the case of sodium carbonate salt mixture, the higher the concentration of the applied sample was, the later was the elution order. Thirdly, the pH of blastocoel fluid is also simulated

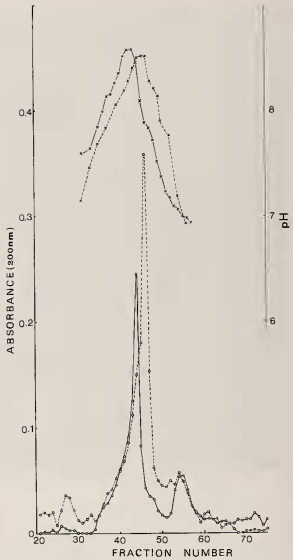

FIG. 5. Chromatography of a mixture of sodium carbonate and sodium bicarbonate.

The equal amount to blastocoel fluid in Fig. 1 (0.3 ml) of 30 mM each of sodium bicarbonate and sodium carbonate (solid line), and 50 mM each of the same compounds (dotted line) were applied. In both cases the salts were solved in 0.1 M sucrose solution. Sucrose was eluted out at fractions of number 53 to 56. The conditions of the chromatography were the same as those of Fig. 1.

by the mixture of equal amount of sodium bicarbonate and sodium carbonate. From the concentration of the total cation (ca. 80 mM) and chlorine ion (ca. 40 mM), actual concentration of each of these salts in blastocoel fluid was supposed to be near to 40 mM. In our preliminary observations of the embryos reared in the dilute Holtfreter's solution containing 0.1 μM antimycin for 24 hr, the blastocoel fluid turned to near neutral (pH 7.6), not but alkaline. This suggests that high concentration of sodium carbonate salts may have some relationship to the product of the respiration of embryos. Kostellow and Morrill have reported

that intracellular sodium moves to intercellular spaces to contribute to the developing blastocoel fluid with no significant change in potassium in the cells during pregastrular stage of *Rana pipiens* [5]. Such a phenomenum is also observed in the case of *Xenopus laevis* [6]. It is possibly supposed that carbon dioxide produced by the respiration of embryonic tissues moves to intercellular spaces and solves in water to combine with sodium to form sodium bicarbonate or sodium carbonate.

By infrared spectrometry, the thrid peak is supposed to contain the small molecular substances of carbonic and aminic structure. Several ninhydrin-positive spots were observed in our preliminary experiments of thin-layer chromatography of the gross specimen of blastocoel fluid. There may be present members of an amino acid group. These small, acidic molecules seemed to buffer blastocoel fluid against salts of sodium bicarbonate and sodium carbonate.

The electrophoretic patterns of the first peak of chromatography and the gross blastocoel fluid were clearly different from those of the homogenate moiety of embryos. The proteins were peculiar constituents of blastocoel fouid, and not cellular fragments contaminated when sucking the fluid from the blastocoel of the embryos. It should be said, however that the largest proteins of 108 and 115 kd were probably the main components of homogenate since they agreed in position in the electrophoresis profile. In any case, blastocoel fluid proved to contain many kinds of protein. Here, when about 0.5 ml of blastocoel fluid was used, at least twenty bands could be counted. The electrophoretic band pattern also showed all the protein components eluted out in the first peak, while the other peaks contained no proteins at all.

Many experiments of early embryonic induction have been hitherto performed by the so-called sandwich method in neutral Ringer's solution as Holtfreter's solution. For the elucidation of the mechanism of primary induction, however, the pH factor may also have to be paid attention to together with inductor itself.

REFERENCES

1 Holtfreter, J. (1933) Nachweis der Induktionsfähigkeit abgetöteter Keimteile. Isolation- und Transplantationsversuche. Arch. EntwMech. Org., **128**: 584–633.

2 Saxén, L. (1961) Transfilter neural induction of Amphibian ectoderm. Develop. Biol., **3**: 140–152.

3 Turin, L. and Warner, A. E. (1980) Intracellular pH in early *Xenopus* embryos: its effect on current flow between blastomeres. J. Physiol., **300**: 489–504.

4 Laemmli, U. K. (1970) Cleavage of structual proteins during the assembly of the head of bacteriophage T4., Nature, **227**: 680–685.

5 Kostellow, A. B. and Morrill, G. A. (1967) Intracellular sodium ion concentration changes in the early amphibian embryo and the influence on nuclear metabolism. Exp. Cell Res., **50**: 639–644.

6 Slack, C. and Warner, A. E. and Warren, R. L. (1973) The distribution of sodium and potassium in amphibian embryos during early development. J. Physiol., **232**: 297–312.